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RAPID COMMUNICATION

Progressive Accumulation of Large Aggregates of Calcium-Containing Polysaccharides and Basophilic Debris Within Specific Thalamic Nuclei After Lithium/ Pilocarpine-Induced Seizures

GHISLAINE F. LAFRENIERE, OKSANA PEREDERY AND MICHAEL A. PERSINGER[1]

Behavioral Neuroscience Laboratory, Laurentian University, Sudbury, Ontario P3E 2C6

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LAFRENIERE, G. F., O. PEREDERY AND M. A. PERSINGER. *Progressive accumulation of large aggregates of calcium-containing polysaccharides and basophilic debris within specific thalamic nuclei after lithium/pilocarpine-induced seizures.* BRAIN RES BUL **28**(5) 825–830, 1992.—Between 30 and 50 days after the induction of seizures by a single injection of lithium and pilocarpine, large aggregates of Nissl-staining material appeared; they occupied up to 35% of the thalamic volume. Both histochemical and atomic absorption analyses indicated elevated concentrations of Ca^{++} (and possibly Mg^{++}) within this substance that was also composed of polysaccharides and nucleic acids. Significant interactions between time since seizure induction and form of the material indicated a progressive accretion of this material from diffusely scattered micrometer granules to large crystalline forms. We suggest this material is composed of endoplasmic reticular debris that is bound by bivalent cations; because the severity of damage exceeds local phagocytic capacity, the material aggregates and then crystallizes. Possible relation to thalamic calcification in neonatal ischemic brains is considered.

Thalamus · Seizure · Calcium · Polysaccharides · Lithium · Pilocarpine · Neuropathology

STATUS epilepticus (SE) is known to produce severe, diffuse damage throughout the telencephalon and diencephalon (4,11,12). Subcutaneous injection of lithium (3 mEq/kg) followed 24 h later by a similar injection of pilocarpine (30 mg/kg) induces SE within about 30 min (4). The state is fatal and most rats die within 24 h. Histomorphology indicates significant cellular necrosis within the thalamus, subcortical limbic structures and entorhinal-piriform cortices (8,12).

However, we found that a single injection of 30 mg/kg of acepromazine allowed the rats to survive. Following a 7–10 day recovery period, the rats displayed deficits in conditioned taste aversion, radial maze learning, and social interaction (12). Spontaneous forelimb clonus occurred occasionally during the subsequent life span of the animal. Despite a relatively normal appearance, the rats proceeded through phases of clearly aberrant behavior. In the course of quantifying the specificity of the brain damage, we noted massive depositions (up to one-third of the cross-sectional area of the thalamus) of darkly stained Nissl substance and crystalline material. Its prevalence increased proportionally with the duration since the induction of the seizures. This study was designed to determine the characteristics of this unusual substance.

METHOD

Subjects

A total of 61 male albino Wistar rats were selected as subjects. They had been injected subcutaneously with 30 mg/kg of pilocarpine approximately 4 h after a subcutaneous injection of 3 mEq/kg of lithium. One h after the injection of the pilocarpine

[1] Requests for reprints should be addressed to M. A. Persinger, Ph.D., Department of Psychology, Laurentian University, Sudbury, Ontario, P3E 2C6.

(about 30 min after the onset of forepaw clonus and rearing), each rat received a single injection of 30 mg/kg of acepromazine to insure survival. They were maintained on a recuperation food and fluid schedule until spontaneous return to appetitive behaviors had occurred.

General Histology and Histomorphology

Fifty-six of the rats were killed by decapitation within 5 postseizure time periods; they were selected on the bases of qualitative changes in behavior. The period and number of rats (in parentheses) that were killed after the seizure onset day were: 1 to 5 days (n = 10), 10 days (n = 15), 15 to 18 days (n = 11), 30 to 33 days (n = 10) and 50 to 95 days (n = 10). Brains were fixed in ethanol-formalin acetic acid (EFA) for over 48 h and then processed. Coronal sections, 10 μm in thickness, were obtained by microtome every 150 μm between the caudal mesencephalon and the anterior commissure. Each section was stained with toluidine blue 0 (9,10).

All of the structures identified by Paxinos and Watson (7) were examined by light microscopy at 100× and 400×. The following scoring system was utilized for each structure: 0 = no damage, Type 1 = neuronal drop out and gliosis, Type 2 = small (less than 10 μm), widely scattered dark Nissl stained material that often showed metachromasia, Type 3 = densely aggregated (> 1 mm) darkly stained Nissl substance (Fig. 1) that often obscured entire structures, and Type 4 = crystalline-appearing dense aggregates of G factor (Fig. 2). The integrated area of all discernable G factor aggregates within serial sections of two rat brains (killed 95 days after the seizure induction) were determined by projecting the slides onto metric graph paper.

Specialized Histochemistry

Serial sections from five different rats that displayed dense aggregations of Type 3 and Type 4 aggregates were stained for calcium, polysaccharides, and nucleic acids. Two methods were used to discern calcium: Alizarine red (fast green counterstain) and silver nitrate (safranine O counterstain). To verify the presence of calcium specifically, some slides were incubated with the metal chelator EDTA for either 10, 30, or 60 min before the calcium stains were applied. The presence of polysaccharides was ascertained by subjecting slides to an Alcian Blue (pH 2.5) and PAS (periodic acid Schiff) reaction, with and without acetylation. A toluidine blue 0 (pH 4.5) procedure was used to determine the presence of nucleic acids.

Atomic Absorption (AA) Procedure

Five male rats in which the lithium-pilocarpine seizures had been induced 70 to 125 days earlier and four age-matched control rats were killed by decapitation. After hemisection of the cerebrum, the right thalamic region was removed by microdissection and prepared for AA analysis; the left hemisection was fixed in EFA for 48 h, processed and sectioned coronally to verify the presence of G factor; these sections were stained as described in the previous section.

The AA specimens were prepared using standard analytical procedures. Each hemithalamus was collected on an acid washed covered petridish and oven dried, partially covered, at 90°C until dehydration was completed as determined by null weight loss (about 1 h). Dried specimens were transferred to covered crucibles and ashed in a muffle furnace over a 25 h period. Ash residues were digested with 10 ml Aqua Regia and 10 ml HNO3 (15.7 N). Elements were redissolved in 3 ml HCl (12 N), filtered into 5 ml volumetric flasks, brought to volume, and refrigerated overnight.

AA readings for Li^+, Mg^{++}, Zn^{++}, and Ca^{++} were completed the following day by a trained technician from a local commercial analytical laboratory. Zn^{++} was selected as a control measure that was considered not be associated with Ca^{++} whereas Mg^{++} was selected because of its frequent co-presence with Ca^{++}. Li^+ was measured to insure that there was no residual from the initial injections.

Statistical Analyses

Repeated measure analyses of variance (MANOVA, SPSSx) was completed for Type 1 to Type 4 damage as a function of the 5 postseizure periods. Parametric and nonparametric ANOVAs were completed between the rats in which seizures had been induced and their controls for the concentration of metals in the brains. Aggregates of general histological scores for various brain structures and means and standard deviations were all completed by SPSSx software on a Vax 4000 computer.

To discern if there was a relationship between the amount of neuronal drop out within the first week after the seizure and the latter occurrence of G factor (Type 3 and 4 damage) within the 45 thalamic nuclear groups (nuclei) that we have consistently evaluated, the percentage of brains (n = 61) that displayed Type 3 and 4 damage was correlated (Spearman rho and Pearson r) with the percentage of brains (n = 61) that displayed cell drop out (8). It is important to indicate that the correlations were completed between the percentages of brains that displayed neuronal drop out and G factor for each thalamic nucleus.

However, we found that the percentage incidence of neuronal drop out in a population of brains is correlated between 0.65 and 0.80 with the average quantitative amount of cell loss within any given nuclear group (8). This suggested that these measures of nominal (0,1) incidence within a population are reliable first order indicators of the actual amount of neuronal loss within the individual.

RESULTS

The structures in which G factor was observed are shown in Table 1. This substance was only found in select thalamic regions and was only conspicuous in rats in which seizures had been induced more than 30 days earlier. The crystalline form was found only in the thalamus of rats that were killed more than 50 days after the induction of the seizure. Integrated cross-sectional analyses for the two brains for which this measurement was completed showed that Type 3 and Type 4 material occupied between 0 and 35% of the total thalamus; the mean for each rat was 5% and 6%. The largest and most conspicuous concentrations of this material occurred within the reuniens, lateral posterior (laterorostral and mediorostral) nuclei, lateral dorsal (dorsolateral) nuclei, the posterior nuclei, and the suprageniculate nuclei.

To determine the dynamic evolution of the G factor over time within the brain, the mean of the absence or presence (0,1) of the anomaly was computed for the 5 thalamic structures that showed the most frequent incidence. Table 2 shows the means and standard deviations (SDs) for the mean scores for each of the four types of damage for rats that were killed during the five periods after the lithium pilocarpine injections. Clearly, the G factor did not emerge until more than 30 days after the seizure induction.

One-way ANOVA indicated no statistically significant differences for Type 1 damage (neuronal drop out) for the 5 groups, $F(4,51) = 0.91$, $p > 0.05$. Because the variance was 0 for rats in groups 1 through 3, only groups 4 and 5 were subsequently analyzed. Two-way ANOVA with one level repeated (Type 2,

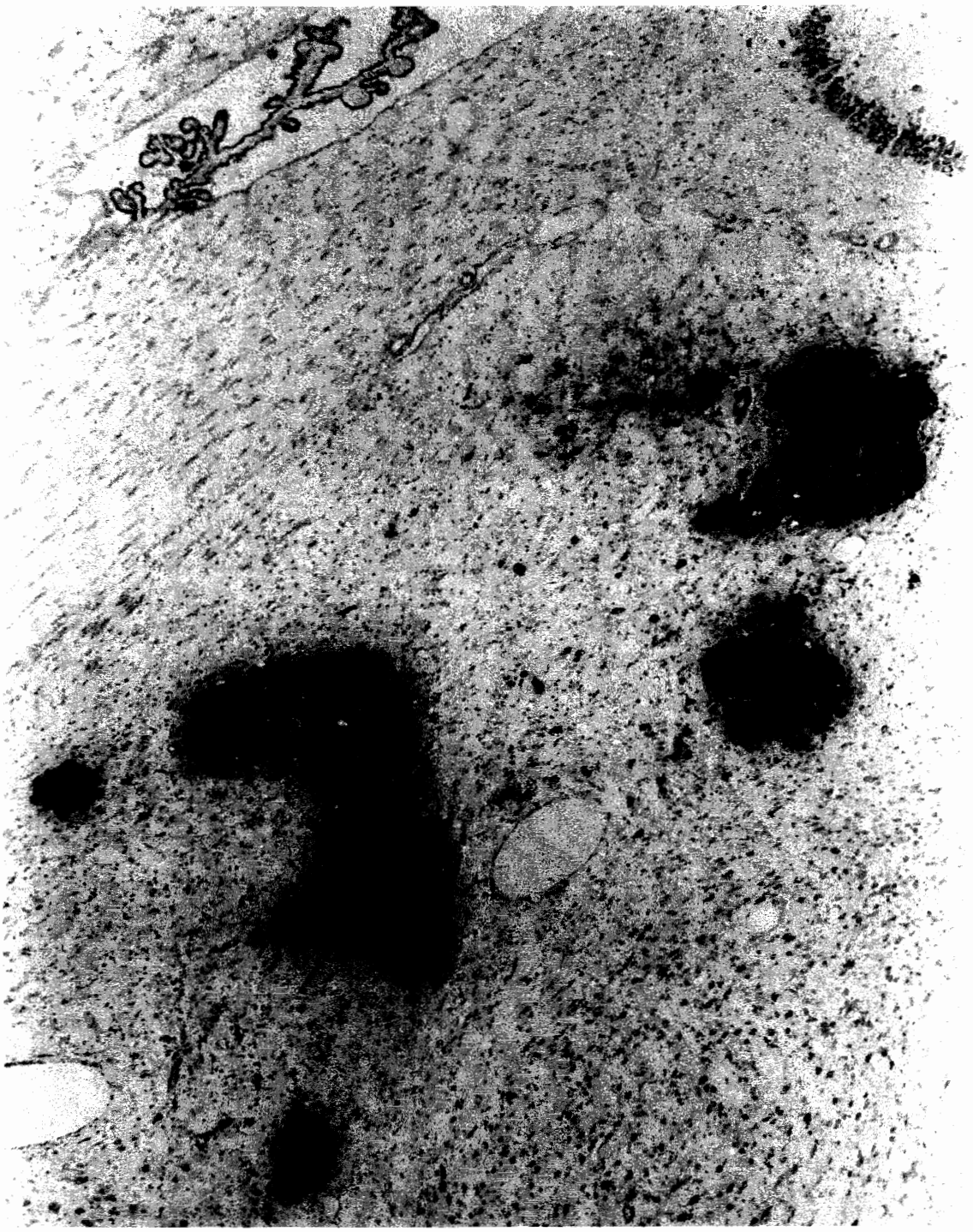

FIG. 1. Distribution of large aggregates (400–700 μm) of basophilic (stained) material within a mid-horizontal section of the thalamus. Smaller clusters of dark-stained material are also shown. The top shows the fimbria-fornix.

Type 3, and Type 4 damage) and one nonrepeated (group) demonstrated no statistically significant differences between groups, $F(1,18) = 0.25$, $p > 0.05$, or types of damage, $F(2,36) = 0.98$, $p > 0.05$. However, there was a highly significant, $F(2,36) = 9.65$, $p < 0.001$, Type × Group interaction. Because of the absence of crystalline (Type 4) material in one group, the repeated measure ANOVA was completed for Type 2 and Type 3 G factor only; again the only statistically significant effect was the Group (time) × Type interaction, $F(1,18) = 11.07$, $p < 0.01$.

These interactions were due primarily to the decrease in small-grained Nissl material but marked increase in the densely aggregated material (Fig. 1) within the brains of rats that were

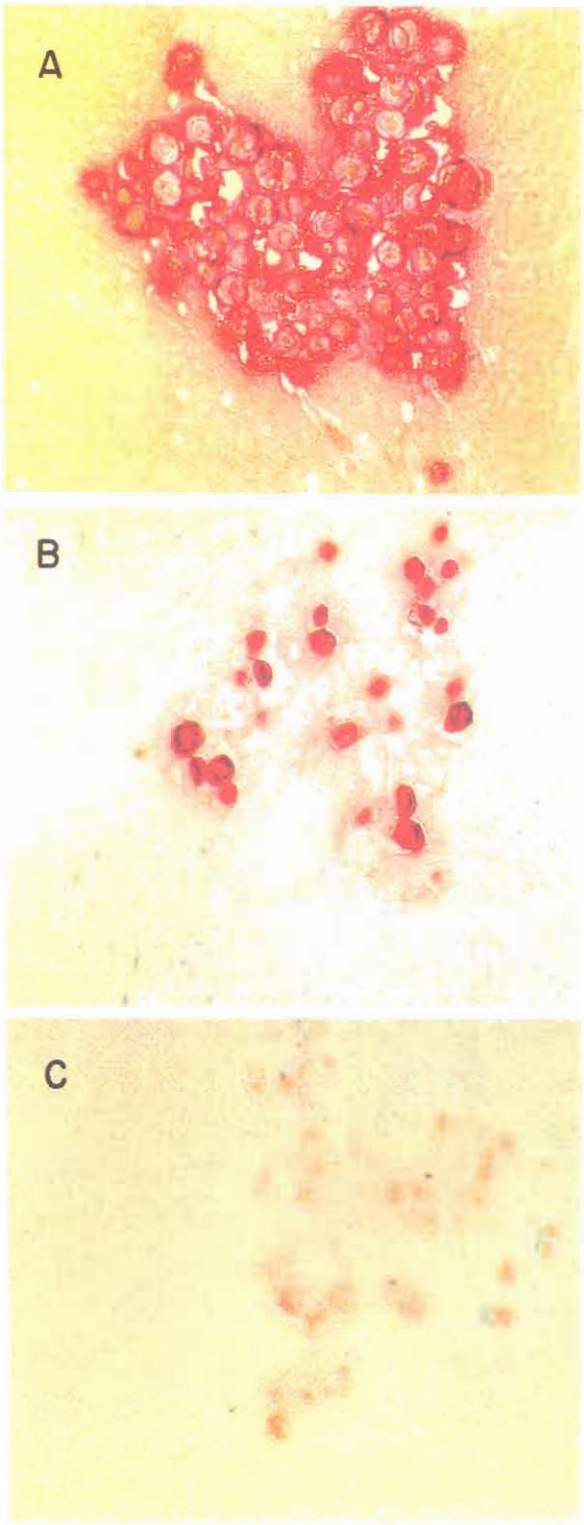

FIG. 2. A) Alizarin Red stained crystalline form of the densely basophilic material from Fig. 1, displaying Ca^{++} presence. B) the Alizarin reaction after preincubation for 10 min with a metal chelator, C) the absence of the Alizarin reaction after preincubation for 30 min with the metal chelator.

TABLE 1

THALAMIC NUCLEI* IN WHICH ANY FORMS OF THE ANOMALOUS MATERIAL (TYPE 3, 4, OR 5) WERE FOUND MORE THAN 30 DAYS AFTER SEIZURE INDUCTION

Structure	%	Structure	%
Po	100	IMD	20
LPLR	65	VL	20
LDVL	45	VPM	20
LPMR	40		
Re	40	PoT	20
SG	40	DLG	20
MDM	30	CL	20
MDL	30	PC	20
VM	30	MDC	15
PF	30	CM	15
AM	25	G	10
MD	25	LPMC	10
LPLC	25	VPL	10
LDDM	25	MGM	10
MG	25	Eth	10
MGD	25	Ang	5
		PVA	5
		PLi	5
		Gu	5
		PT	5

Values reflect the precentage of brains (n = 20) that contained this material.

* Abbreviations after Paxinos and Watson (7).

killed +50 days after the seizure. The exact opposite pattern (predominate small-grained material and very little aggregated material) was displayed by the rats that were killed 30 days after the seizure induction. The crystallized material only appeared in the rats that were killed more than 50 days after the seizure induction.

The scattergram between the percentage of brains that showed (Type 1) neuronal drop out within 1 week after the seizure and the percentage of brains that showed G factor for the 45 thalamic groups is shown in Fig. 3. The Pearson r was 0.63 whereas the rank order correlation was 0.73. An inflection point for the occurrence of the G factor occurred when the percentage of brains that displayed neuronal dropout within an area during the first week exceeded 40%.

TABLE 2

MEANS AND SDs FOR THE PERCENTAGE OF BRAINS THAT DISPLAYED TYPE 1, 2, 3, AND 4 WITHIN FIVE THALAMIC NUCLEI

Postseizure Time	Neuronal Loss		Diffuse Granules		Large Aggreg.		Crystal Forms	
	M	SD	M	SD	M	SD	M	SD
1–5 days*	80	33	0	0	0	0	0	0
10 days†	78	22	0	0	0	0	0	0
15–18 days*	70	27	0	0	0	0	0	0
30–33 days*	81	12	43	30	7	21	0	0
50–95 days*	68	18	3	7	31	42	23	23

* n = 10.

† n = 11.

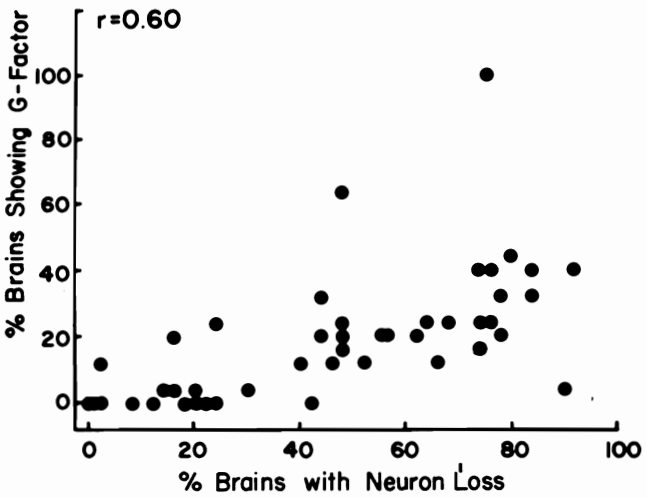

FIG. 3. Percentages of brains that displayed G factor or mineralization (>30 days after seizure induction) as a function of percentages of brains that displayed neuronal drop out within the first week after seizure induction for 45 thalamic nuclei.

The histochemical stains indicated that the G factor was composed of Ca^{++}. Both the alizarine and silver nitrate procedure displayed a strong positive reaction. Incubation of the brain sections within EDTA before staining produced a duration-dependent decrease in the amount of specific staining. Fig. 2 demonstrates the loss of the alizarine red reaction within the crystalline form of G factor after various durations of incubation with the calcium chelator.

The means and SDs for the concentration of metals (μg metal/g of dry weight) for the control rats were: Li: 23 (SD = 18), Mg: 550 (SD = 27), Zn: 478 (SD = 290), and Ca: 427 (SD = 33); the means and SDs of the concentrations of these metals within the hemithalamus of rats in which the seizures had been induced more than 60 days previously were: 27 (SD = 21) μg/g, 735 (SD = 138) μg/g, 1063 (SD = 506) μg/g, and 1185 (SD = 702) μg/g. Bartlett's box for homogeneity of variance demonstrated that there was a significant increase in the variability of Ca^{++} within the epileptic group, $F = 13.53$, $p < 0.001$. Consequently, nonparametric Kruskal–Wallace tests were performed. They verified the significant increase in the thalamic concentrations of Ca^{++} ($\chi^2 = 6.00$, $p < 0.01$, and Mg^{++} ($\chi^2 = 6.0$, $p < 0.01$, for the rats in which seizures had been induced; neither Zn nor Li showed statistically significant group differences. Pearson correlations between all four metals indicated that only Mg^{++} and Ca^{++} were significantly correlated ($r = 0.91$).

Histochemical procedures that were designed to establish the content of the material in which the Ca^{++} (and Mg^{++}) was embedded revealed a PAS positive reaction, even after acetylation. These results indicate that polysaccharides were present within the G factor but that they did not contain 1, 2-vic glycol reactive groups. That the G factor reacted positively to the toluidine blue O stain (pH 4.5), strongly suggests the presence of nucleic acids, however, the participation of some phosphate-bearing proteinaceous moiety cannot be completely excluded at this time.

DISCUSSION

Sustained seizure activity is well known to produce neuronal death. When this activity is maintained within large volumes of the brain, the subsequent neuronal drop out must involve millions of cells. A typical response of fragmentation within the endoplasmic reticulum is the formation of innumerable vesicles (5). The sudden release of cell organelles and fragments into the extracellular space, that follows the catastrophic cell loss, would exceed the brain's phagocytic capacity. We suggest that over time these fragments accrete and that the process is facilitated by the two most concentrated cations: calcium and magnesium.

There is compelling evidence that the large aggregates of basophilic staining material within the thalamus are cell fragments. The correlation between the severity of neuronal drop out within 45 different thalamic nuclei during the first week after the seizure induction and the incidence of G factor in those same nuclei was strong (rho = .73). In addition, the amorphous G factor, whose integrated area ranged between 0% and 35% of a section, was especially concentrated at thalamic levels that exhibited the most extensive neuronal loss.

Qualitative inspection of the thalami of rats that were killed between 1 and 200 days after the induction of seizures supports the concept of aggregation. Within the first post-seizure week, irregular shaped grains whose sizes ranged from those of mast cell granules (10) to small glial nuclei were apparent. Many of these granules revealed metachromasia, indicating the presence of mucopolysaccharides. As the diffuse "bluish field" disappeared, larger and larger aggregates of amorphous G factor appeared.

More compelling evidence of an accretion process was indicated by the highly significant quantitative interaction between the postseizure time and the incidence of amorphous and crystalline material. The decrease of the former and the increase in the latter in the same nuclear groups demonstrated that both are the same material that is undergoing both physical and chemical changes. The amorphous material was not apparent during the first two to three weeks after the seizure induction.

The presence of a threshold of damage before the occurrence of G factor supports the concept that it accumulates when the neuronal necrosis exceeds the limits of normal phagocytic processes. No G-factor occurred in a nuclear region unless the incidence of neuronal drop out in the brains of rats that were killed during the first week exceeded 40%. Above this critical mass of fragmented material, other processes dominate.

Although a logical counterargument is that the occasional spontaneous seizures that follow recovery from SE drive the process by producing more cell death, this explanation is unlikely. Quantitative analyses (8) indicate that most of the neuronal death occurs within the first week. In fact, most of the actual damage occurs within the first 10 hours and then simply becomes more obvious during the next 100 hours.

We hypothesize that one process maybe the accretion of cell fragments, particularly the reticulum, into larger and larger aggregates that are cemented by Ca^{++} and Mg^{++}; the result is focal mineralization of the brain. The significant elevation of Ca^{++} and Mg^{++} as determined by AA and the specific histochemical staining for Ca^{++} supports this hypothesis. Concomitant presence of mucopolysaccharides, which are associated with the extracellular membrane and exhibit polyanionic properties, could facilitate the binding of Ca^{++} and Mg^{++} cations. One candidate could be calsequestrine, a protein that can bind as many as 43 Ca^{++} per molecule (6); it is a major component of the endoplasmic reticulum. The fractionation of endoplasmic reticulum into a myriad of vesicles could increase the relative surface area of calsequestrine and thus facilitate binding of ambient Ca^{++}.

Such a process would require powerful bonding forces that would attract cell fragments. One possibility could involve the Ca^{++}/Mg^{++} polysaccharide matrix. Simple calculation indicates

that if the concentration of Ca^{++} (6) and Mg^{++} is increased by a factor of 2 and the average cross-sectional area of G factor in the thalamus is 5%, then the concentration of these ions must be 21 times greater than normal. This molarity would exceed the typical crystallization values required for (example) calcium carbonate. Therefore, these cations must be sequestered by some other extremely high affinity protein that displays substantial electrochemical potential. Such a substance may explain the unique appearance of the mineralization within the thalamus.

Comparable damage occurs within the limbic cortices, amygdala and hippocampus yet no G factor has been observed within these regions. Although this could simply reflect the differential capacity within these regions to generate phagocytic gliosis, specific differences in high affinity proteins should not be ignored. For example, paralbumin, which is found in a subpopulation of cortical GABA neurons, displays extremely high Ca^{++} affinity (1). A similar protein within thalamic space may be responsible for the preferential deposition of the G factor.

Considering the prevalence of brain proteinases, however, a Ca^{++}-binding protein would be expected to be slowly but ultimately digested. As a consequence, unbound and oversaturated concentrations of Ca^{++} would crystallize into granular form. This process may explain the greater incidence of crystalline granules after the formation of amorphous G factor. If this developmental sequence is correct, then the actual volume of the amorphous material should decrease as the crystalline material increases and occupies much less volume. Qualitative examination supports this conclusion.

Calcification or ferrugination (mineralization) of the human brain occurs in several disease states (2,3). Intracellular accumulation of Ca^{++} and iron granules occurs around microhemorrhages. In addition, the occurrence of extracellular mineralization in context of severe cell necrosis, is best documented in neonates and infants (3). This form of calcification is exclusively located in the thalamus and adjacent basal ganglia. About two to four weeks are required after the initial ischemic insult before the calcification becomes evident by both CT and postmortem analysis.

Our results are remarkably similar to infantile thalamic calcification. Until recently, rats in which lithium/pilocarpine seizures had been induced died within 24 h. The discovery that acepromazine reduces mortality and facilitates longevity has allowed quantitative documentation of the development of the interesting phenomenon of thalamic mineralization.

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